**Dear Dr Nguyen and dear reviewer,**

**Thank you very much for your kind and thorough revisions. We appreciate your input and tried to address all your comments. Please find our changes in the manuscript and our remarks in blue in this document:**

**Editorial comments:**

Please maintain the current formatting throughout the manuscript. The updated manuscript (54861\_R1\_050916.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.  
  
1. Formatting:  
-Section 1 heading – Please use a single statement/phrase as a heading.

We changed the header to: **Description of the offline preparation and sample generation of a D5 deletion protein.**

-Please define all abbreviations at first occurrence (ie LB, etc.).

Thank you for the remark, we defined them in the text now.

-It is only necessary to define the abbreviation DTT once. Please use the abbreviation after it is first defined (1.11).

We removed the unnecessary full terms.

-Please include all software used in the materials table.

Please find them in the table.

2. Grammar:  
-Please copyedit the manuscript for numerous grammatical errors, which is required prior to acceptance. Some errors are described below.  
-Please use American English throughout the manuscript. For instance, “analyse” should be “analyze”.

It is changed.

-1.2 – “Express D5323-785 in 12 L LB with Ampicillin bacterial culture”

It is changed.

-Please do not capitalize “ampicillin”. It is not a proper noun.

It is changed.

-1.5, 1.8, 1.13, 1.17, 2.2.6, 2.2.7, 3.2 – Please use “Check” or similar rather than “Control” here and look for similar uses throughout the manuscript. “Control” is not used correctly.

It is changed.

-2.2.11 – “software start the”

There was an and missing.

-Line 509 – “is a required”

The a is removed.

-Line 526 – “as method of choice and work”

The “and work” part is removed.

3. Additional detail is required:  
-1.1.7.7 – Are antibiotics used? If so, at what concentration?

Information is included in the text.

-1.1.8 – What concentration of ampicillin?

Information is included in the text.

-1.2 – Are bacteria grown at 18 degrees the entire time?

No, first they were grown at 37°C. We clarified that in the text.

-1.3 – What are the sonication settings?

The settings depend largely on the machine and probe used in each laboratory. Still, we gave our settings and included the machine in the material list.

-2.1.1 – What buffer is used?

The gel filtration buffer.

-3.1 – How is ISPyB used to do this?

We hope the changed text elucidates it.

-3.3.2 – How is scaling performed?

By pressing the Scale button in the program

-3.4 – How are the files merged?

By pressing the Merge button in the program

-3.5, 3.6 – How are these determined? Detail is required to include these in the video.

We have added which parts of the software need to be used. Details on how exactly to determine a “good” distribution function are outside the scope of this paper (typical at least a few paragraphs in SAXS textbooks).

4. Unnecessary branding should be removed:

-1.3 – benzonase

We removed the name and used the generic: DNase.

-Please remove all trademark symbols from the materials table.

-3.2, 3.3, 5.1, 5.3 – PRIMUS

We replaced it.

**Reviewers' comments:**

**We would like to thank all the referee once again for their insightful comments that helped us to improve the paper. Please find changes, explanations and comments to each of your raised points.**

**Reviewer #1:**  
*Manuscript Summary:*  
The topic covered in the current manuscript is very significant to the future of small angle X-ray scattering (SAXS) of macromolecules and macromolecule complexes. Having chromatography systems linked to the beam line set up will surely aid in data collection for transient protein complexes and partially aggregated samples. Here are some suggestions to improve the manuscript for the video.  
  
The protocol presented by the authors although quite detailed is very specific to the BioSAXS BM29 beam line at ESRF at Grenoble, France. The title suggests the protocol will be more general. A more general protocol will better serve the scientific community at large.

We added a paragraph to the discussion

For example: Explain about the reason and probable challenges one may face while adopting such systems at other beamlines.

We added a paragraph to the discussion.

Why choose step-wise gradient over continuous gradient? PS: Figure B show a continuous gradient while text insists in use of step-wise gradient.

The figure is as described in the text the offline profile of the protein, where we used a linear gradient, on with we based later our choice of gradient steps.

Detailed protocol for protein purification is not relevant to the current work.

We agree that it is not absolutely necessary but we were specifically asked to put it in by our first editor.

Maybe worthwhile to mention an overall range of dilution factors and minimum volumes required when performing size exclusion chromatography linked SAXS.

As both the dilution factor and the minimum volumes required depend on the type of column and the sample (the authors have seen injections volumes as low as 10 µL and as high as 1 mL), we decided not to add this kind of information.

What is the rationale behind the automated buffer profile generation?

We are not sure what the referee is referring to with automated buffer profile generation. If he refers to the automated buffer subtraction in the SEC experiment, we added a relevant citation in 2.1.14 and 2.2.19.

When would one chose Ion exchange versus size exclusion SAXS?

In our introduction we describe the different use of the two chromatographic methods (“By using the charge, the separation of similar types and sizes of molecules, which would otherwise be difficult to separate, can be made routinely with IEC. Additionally IEC has the advantage to be able to deal with diluted samples, avoiding concentration steps, which carry the potential risk of denaturating the protein. Unfortunately, as the charge distribution is highly sample dependent, IEC requires optimization regarding the pH and the salt concentrations [13](#_ENREF_13),[14](#_ENREF_14).

For many proteins, which are difficult to express, purify or both, only low quantities of sample are available to study. It is important to be efficient and minimize the number of purification steps and therefore the losses. For this reason, the last purification step is online directly prior to SAXS data acquisition in order to increase the likelihood to collect a good data set.”)

We think that a more detailed discussion would be too long in the context of this publication and can be found easily in text books or online.

Furthermore the 3rd paragraph of the discussion (“In practice, in first instance, SEC-SAXS is likely to be used as method of choice for most macromolecular samples. Still many purification protocols require a prior IEC step due to the presence of contaminants or aggregation. Given that each concentration and chromatography step comes along with a loss of sample (estimated 30-50%) and time, direct IEC-SAXS is advantageous. “) mentions our reasoning again.

We added “For samples which cannot be purified by SEC, be it due to the presence of similarly sized “contaminants” or because they severely aggregate at the necessary concentrations, IEC-SAXS would always be the better suited approach. Also, the higher flow rates supported by many IEC columns can help to reduce the transit time between purification and measurement.”

*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #2:**

**Thank you very much for your comments. They are highly appreciated.**

*Manuscript Summary:*  
The authors of the manuscript (JoVE54861) report the practical application of SEC- and IEC-SAXS at ESRF BM29 using the vaccinia virus protein D5. The protocol covers from gene cloning to protein purification of D5323-785 protein, a deletion mutant of D5, as well as SAXS experiments and analyses. A striking feature of this manuscript is that the authors devise and implement a strategy for background adjustment of IEC-SAXS data. An IEC-SAXS data of D5323-785 protein was reasonable and comparable to SEC-SAXS data.  
However, the readability of the manuscript is very hard particularly in protocol section. Below are major and minor comments for the author's consideration that absolutely need to be addressed before publication.  
  
*Major Concerns:*  
1. (overall in protocol session) Add product name listed in table, especially if manufacturer's protocol is referred.

We agree that it would be easier but JOVE explicitly asked us to remove all product names. They can be found in the table of material.

It would be a good idea to add a short title in each paragraph as much as possible (particularly secession 2.2 is hard to understand).  
For example, 1.9 (line 203) could be:  
1.9 [Ni-Affinity Chromatography] Equilibrate the Ni-column (His-Select HF Nickel Affinity Gel) in binding buffer. Let the protein solution….  
2. (SEC- & IEC-SAXS parts in protocol session) Model name of HPLC system (also software name and its version) should be described in the text since there are some system-specific terms in protocol.

As mentioned above we had to remove all product names. Regarding titles in 2.2, it is indeed the workflow as it is described here and we rather feel that it would interrupt the flow by adding more titles.

The details of the column used for D5323-785 protein are also missed in text, especially column volume. It would be a good idea to repeat the column and buffer information even if the details are duplicated.

We agree that it would be easier but JOVE explicitly asked us to remove all product names. They can be found in the table of material.

3. (Line 269) What is "the number of counts"? Transmission intensity measured by photo diode? Need to describe the details.

We clarified “Write down the number of counts given by the detector in the summed intensity plot”

4. (2.2.12, 2.2.18 & Fig 1B) If I understood the protocol correctly, the protein was eluted by 2% step gradients whose volume is 2.5 CV/ gradient. The gradient used for Fig 1B doesn't look like 2.5CV step if the column volume is 1.3 ml. Figure 1B should be replaced with the profile at IEC-SAXS experiment (not that of purification). Otherwise add such a profile in addition to Figure 1B. The profile should be magnified around elution peaks (e.g. 10-40mL).  
Salt concentration of SAXS data used for modeling should be mentioned as well.

We added a figure showing the actual profile of the IEC SAXS run (1C) and precised the explanation of the steps we used.

5. (Line 550-) Two SAXS curves are obviously different as the authors mentioned. The authors assumed that the difference mainly comes from the different salt concentrations. In my opinion, I think that protein conformation and/or SAXS profile are more susceptible to high concentration of glycerol (e.g. >5%) than salt concentration. I guess that difference of salt concentration between SEC and IEC is probably less than ~30mM. It is hard to imagine that SAXS profiles are dynamically changed like Figure 1C and 1D by small difference of salt concentration. High concentration of glycerol can alter inter- and/or intra-molecular interactions and changes the degree of hydration shell which is ideally taken account during SAXS modeling.

We clarified it in the text in both cases SEC and IEC SAXS we used 5% glycerol in the buffer. Another slight difference is that the proteins were prepared on different days.

After careful evaluation of the data we agree that the salt concentration is less likely to cause the differences, but maybe the differences in the sample preparation, in the time between purification and measurement (IEC is faster) or, less likely, to a contaminant in the SEC purified sample.

A sentence is added to the text.

The authors mentioned that D5323-785 protein measured by IEC-SAXS is more compact than that of SEC-SAXS, based on Rg and Dmax. However, Porod volume is opposite. Together with above, more careful discussion should be required.

The differences between the curves are quite small and do not affect the conclusions on the shape of D5323-785 as mentioned in the text.

6. (Line 555-) The chi square value is the error-dependent value. It is irrelevant to compare experimental SAXS curves based on chi or chi square value by model fitting. Remove the sentence.

Done.

7. (IEC-SAXS) The zero-extrapolation is not performed in this protocol. Effect of interparticle interactions would be remaining in the profile. Also this procedure tends to have over-subtraction when sample is very elongated or flexible. Accordingly, careful validation, ideally by different methods, should be required. The authors should discuss such potential risks in text.

The zero-extrapolation was not explicitly mentioned in the manuscript. However, just as for SEC-SAXS we verified a stable radius of gyration (based on a preliminary buffer subtraction) before proceeding. We now explicitly mention this in step 5.2

*Minor Concerns:*  
8. (overall) Name of all software (beamline control, etc) should be also indicated to clarify currently working software/program. "beamline control system" and "beamline control software" are same? Also need to indicate the version of ATSAS package.

We unified the term and the version can be found in the material table.

9. The title of the manuscript sounds somewhat generic. The words of both "D5" and "ESRF BM29" would be included in the title.

We did not change the title of our manuscript given that we believe that D5 and BM29 are served as an example to show that the method is working and that this can be implemented on different SAXS beam lines.

10. No word of "SAXS" in short abstract.

We included SAXS in the short abstract.

11. (Line 73) Need reference regarding separation of SEC. In general, it is impossible to separate monomer and dimer by SEC. "10% difference" sounds weird.

There are plenty of examples for the separation of mono and dimers by SEC in the literature. See for example J.A.P.P van Dijk, J.A.M Smit (2000)

12. (Line 141-) It is awkward that individual protocol is only provided for the competent cell (Top 10?). Change to "the manufacture's recommendation" in the same fashion?

JOVE explicitly asked us to remove all product names. They can be found in the table of material. The paragraph 1.1.11 was elaborated.

13. (Line 189) 1x Laemmli running buffer. Convert to concentration.

It is changed.

14. (Line 216-) 1.14. Need rewording.

We hope is now better understandable.

15. (Line 288) 3.10 ⎝ 2.1.10?

Sorry, we do not understand the meaning of the question.

16. (Line 365) What kind of detector? UV detector?

All detectors.

17. (Line 425) paragraph 3.8 and 3.9 should move to paragraph 4. Accordingly, the title of paragraph 3 needs to be changed. Then paragraph 4 should be something like "ab initio modeling".  
Title of paragraph 5 would be "IEC-SAXS data reduction and analysis ( or modeling)".

We followed the excellent suggestion of the referee.

18. (Line 171 and175) Missing a step of centrifugation? Need to mention speed and time. Also those values should be mentioned in all centrifugal places.

We added the procedure to the step and added the speed and time to 1.1.7.6

19. (Table 1) The number of flux is correct?

No, the formatting was wrong. It should be 1012.

20. (Figure 1B) Why does vertical axis have arbitrary unit?

Because the units in the plot are arbitrary (albeit with a known scaling factor to cm-1).

*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**

**Last but not least thank you so much for your help in improving this manuscript!**

*Manuscript Summary:*  
This manuscript describes SAXS data acquisition and analysis with on-line, pre-SAXS measurement SEC or IEC purification. An example of the vaccinia virus protein D5 is given to demonstrate the performance of the two different purification options. Overall it can be a valuable contribution and the comparative analysis of the two methods and their results is instructive.  
  
The points below are essential to be address adequately in a revised submission.

1. The first sentence of the abstract overstates the importance of BioSAXS and should be pulled back some to be more in line with the language of first few sentences of the introduction, which acknowledges it as a powerful (as opposed to the arguable "indispensable") technique and provides an accurate description of the information inherent to the scattering data (which falls short of "the solution structure" but does provide solution structural parameters that can be powerful constraints or tests).

We changed it to powerful.

2. Dilute solution conditions are not "close to physiological" - they can mimic certain important parameters such as ionic strength, pH etc. Paragraph 1 needs to be corrected on this point.

It is changed to: The technique does not require crystals and the macromolecule stays in solution, thus can be kept in conditions mimicking certain important parameters of the cell such as ionic strength, pH etc..

3. In paragraph 2 the conditions that can lead to uninterpretable data are noted, but more significantly these conditions can lead to biased data where the bias is undetected without great care in the data reduction and analysis. Hence the data are interpreted but give inaccurate results - e.g. subtle aggregation or inter-particle interference. This should be noted.

We added this additional issue to the paragraph.

4. Reference 12 is "in submission" it seems important to this paper. Its acceptance and hence availability to the reader of this paper I presume would be important.

We are of hope that at the time this manuscript will be published the submitted paper will be available to the public (the paper is accepted).

5. lines 84-85; are the authors saying dilution can cause denaturation? Is this common? Can they clarify?

No, what we say is that IEC has the advantage to be able to deal with diluted samples without a potentially denaturating concentration step. This means that during the concentration step samples might denature and yes, that happens often. For example; some protein precipitate on the membranes of concentrators or if they are too concentrated. We changed the formulation.

6. line 417 point step 3.7 states that one should "crop the data" before the beginning of the Guinier region. This is a potentially dangerous step as it can minimize without actually removing the effects of subtle but significant aggregation or inter-particle interference. While it is a common practice, it is one that should not be approach blithely as routine. There is no objective way of determining "the beginning of the Guinier region" - rather it takes careful consideration of the scattering profile overall. From this point the paper proceeds with the automated ATSAS data processing and interpretation. This automated pipeline might be helpful for getting some measure of feedback on your data during the experiment, but given the many subtleties in SAXS data, the requirement for careful post experiment analysis and validation cannot be underestimated. This point needs to be made very clear at this stage in the protocol and in the discussion.

We agree with the reviewer that is a common practice that requires some kind of experience and given that we use the p(r) we removed the paragraph from the paper.

7. line 485 "model free" is a bit misleading. The data in Table 1 are derived from the Guinier model, P(r) model, and a model for the high-q scattering form Porod all of which make assumptions about the shape and surface of the scattering particle. It is correct to say that the results are independent of any specific shape or atomistic model and can be derived directly from the scattering data.

The only assumptions for Guinier and P(r) are that the system is biphasic and that the Guinier range is accessible. Hence, the values themselves are not based on any model (although their interpretation may be). For the Porod volume, there is the additional globularity requirement, but even this is not a “model”. We did however add the term “invariant” to the text to be more specific.

8. A significant limitation of the combined SEC-SAXS and IEC-SAXS method is that one does not know the protein concentration in the sample at the point of measurement, which precludes using I(0) to determine the molecular mass of the scattering particle, an essential validation parameter given the strict requirement for monodisperse particles of the same size for accurate structural interpretation. To cope with this limitation, the Porod volume and now the somewhat arbitrary half of the volume of the dummy atoms in the bead modelling is used by many. The Porod volume is fraught by its dependence on the scattering invariant which requires integration from 0 to infinity, and hence assumptions about the high-q behavior beyond the measurement range and in the range where background subtraction errors and flexible/unfolded regions in the structure can be a problem. This issue should be acknowledged and appropriately noted.

We added a paragraph addressing this issue to the discussion.

9. The authors talk about "good" background subtraction and need to describe how they assess "goodness." Background subtraction at increasingly powerful synchrotron sources is notoriously becoming more difficult and common practice in batch measurements is to measure solvent backgrounds before and after the sample, subtract one or the other and average of both and by some criteria decide which is best. Given the contributions of internal density fluctuations in the high-q scattering, how is a "good" background assessed. It would be valuable to describe here given the emphasis on improved background subtractions - why are they good with this method and how do they compare to the batch mode.

We agree with the referee that the question of what constitutes a correct background subtraction is indeed complex. For this reason, we actually subtract different backgrounds in step 5 and only trust results robust against background subtraction. We therefore do not claim to have an improved background subtraction.

The answer to the question of what would constitute a “good” background in our case is actually provided in the note of step 5.3: “There should be no systematic offset between the average from the peak and the buffer.” The reason for this is that while the contribution of the density fluctuations is often not negligible, it generally does not give a q-independent signal.

As we don’t think that generalized statements about which method allows the best background subtraction are possible, we would like to refrain from adding such a discussion.

10. lines 552-555 The authors need to consider if the difference in the results reflect the fact that the SEC sample is contaminated with a small amount of aggregate by the time it reaches the SAXS measurement cell. This seems counter-intuitive as the I(0) data indicate the SEC sample is more than and order of magnitude more concentrated than the IEC sample. The authors should comment on this.

Based on the forward scattering, the SEC sample is 10 times less dilute than the IEC sample. Hence, one would expect the IEC sample to be more prone to aggregation. We did extend the discussion of possible reasons for the observed differences.

11. In regard to 10 above, what is the distance and time taken for transit from the SEC or IEC to the SAXS sample chamber? This would potentially be where samples highly prone to aggregation could start to aggregate, and hence point 8 above is more significant as with accurate concentration determination (possible with careful A280 measurements and accurate extinction coefficient assessment) one can measure molecular masses reliably with an accuracy of 5-10%. Obviously this paper cannot solve this problem, but it should be acknowledged as a limitation and if people are working on solutions, referenced.

We have added the difference in transit time as one reason why one might choose IEC in the discussion. We also address this as a possible source for the observed differences between the two curves.

12. In the table it says "monomeric Mr" when it seems it should be "hexameric Mr"?  
  
It indeed should be and is now corrected.

While there are many detailed points made above, they are made with the view of improving the paper and contributing to strengthening confidence in SAXS as a valuable structural biology technique when used with care.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A